TECHNICAL MANUSCRIPT 139

GROWTH OF SELECTED ARBOVIRUSES IN SERUM-FREE SUSPENSION CELL CULTURES

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GROWTH OF SELECTED ARBOVIRUSES IN SERUM-FREE SUSPENSION CELL CULTURES

Henry R. Tribble, Jr.

H. J. Hearn, Jr.

S. C. Nagle, Jr.

W. T. Soper

Medical Bacteriology Division
and
Virus and Rickettsia Division
DIRECTOR OF BIOLOGICAL RESEARCH

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ABSTRACT

Information on the feasibility of serum-free suspension cultures for the production of viruses is scant. Evidence that cells, grown in serum-free suspension systems, are capable of supporting the growth of Venezuelan equine encephalomyelitis (VEE) and yellow fever viruses to titers that equaled or exceeded other in vitro systems has been obtained. HeLa, L, cat kidney, and chick embryo cell lines were grown in either lactalbumin hydrolyzate (LAH) medium or a chemically defined medium. These media consisted of inorganic salts, vitamins, glucose, and Methocel to which either LAH or 13 or 19 amino acids were added. All cell lines were grown in their respective media for at least a month prior to infection in 100-milliliter serum bottles (30 ml medium per bottle) on a New Brunswick shaker at 35°C. Maximal virus titers of 10^8 to 10^9 mouse intracerebral (MIC) LD_{50} per ml of VEE virus were obtained 24 hours after infection with concomitant cell lysis in the serum-free cultures of all four cell lines grown in lactalbumin hydrolyzate. Similar results were obtained in cat kidney, L, and chick embryo cultures grown in media containing 19 amino acids and in L cell cultures grown with 13 amino acids. Yellow fever virus was also successfully propagated to titers of 10^7 to 10^8 $MICLD_{50}$ per ml in HeLa and chick embryo cells in 4 to 5 days with these media. The high degree of selectivity of such cell systems for viral populations that have widely divergent genetic characteristics is discussed.

I. INTRODUCTION

The maintenance of continuously cultured animal cells* in suspension cultures that could be used for growing virus ordinarily requires the periodic addition of serum. Three major disadvantages of employing serum for this purpose, however, are that it offers the possibility of introducing (1) contaminating viruses or other microorganisms such as pleuro-pneumonia-like organisms, (2) antigenically active foreign proteins, and (3) protein or metabolic substances in undefined quantities. Information on the feasibility of using serum-free suspension cultures for the production of viruses is scant, i.e., the nutritional requirements for viral growth and the characteristics of viral populations grown in such systems have not been defined. It is the purpose of these studies to provide data in these areas. Venezuelan equine encephalomyelitis (VEE) virus was selected for studying viral growth in serum-free suspension cultures. It has been shown to be capable of replication in a wide variety of cell lines grown in serum-containing media and this viral growth can be assayed by several methods. Results of growth studies on a highly fastidious agent, yellow fever virus, will also be presented. In addition, this report will include evidence that serum-free suspension systems are selective for viral populations possessing widely divergent genetic characteristics.

Two serum-free growth media were used in these experiments. The first, presented in Table I, consisted of lactalbumin hydrolyzate and glutamine as nitrogen sources, vitamins, carbon sources, salts, antibiotics, and the additives Methocel, insulin, and phenol red. This will be referred to as the LAH medium. The second consists of amino acids (some essential and some nonessential for the growth of these cell lines), salts, carbon sources, vitamins, antibiotics, and the same additives as in the first medium. This will be referred to as the NEAA medium and is shown in Table II.

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* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

TABLE I. LACTALBUMIN HYDROLYZATE MEDIUM FOR SUSPENDED CELL GROWTH

Component	Concentration, mg/l	Component	Concentration, mg/l
Nitrogen Sources:		**Salts:**	
Lactalbumin hydrolyzate	2500	NaCl	7400
L-Glutamine	300	KCl	400
		$NaH_2PO_4 \cdot H_2O$	100
Vitamins:		$NaHCO_3$	300
		$CaCl_2 \cdot 2H_2O$	265
D-biotin	1.0	$MgCl_2 \cdot 6H_2O$	275
Choline·Cl	1.0		
Folic acid	1.0	**Antibiotics, etc.:**	
Niacinamide	1.0		
Ca pantothenate	2.0	Methocel 15 cps	1000
Pyridoxal·HCl	1.0	N.P.H. insulin	200 units/l
Thiamine·HCl	1.0	Streptomycin	100 mg/l
i-inositol	1.0	Penicillin	100,000 units/l
Riboflavin	0.1	Kanamycin	100 mg/l
B_{12}	0.002	Phenol red	10 mg/l
Carbon Sources:			
Glucose	1000		
Sodium pyruvate	110		

TABLE II. CHEMICALLY DEFINED MEDIUM FOR SUSPENDED CELL GROWTH

Component	Concentration, mg/l	Component	Concentration, mg/l
Amino Acids (essential):		**Salts:**	
L-Arginine·HCl	100	NaCl	7400
L-Cysteine·HCl	75	KCl	400
L-Histidine·HCl	60	$NaH_2PO_4 \cdot H_2O$	100
L-Isoleucine	150	$NaHCO_3$	500
L-Leucine	300	$CaCl_2 \cdot 2H_2O$	265
L-Lysine	300	$MgCl_2 \cdot 6H_2O$	275
L-Methionine	60		
L-Phenylalanine	120	**Carbon Sources:**	
L-Threonine	135		
L-Tryptophan	60	Glucose	1000
L-Tyrosine	120	Sodium pyruvate	110
L-Valine	150		
L-Glutamine	450	**Vitamins:**	
Amino Acids (nonessential):		D-biotin	1.0
		Choline·Cl	1.0
Glycine	60	Folic Acid	1.0
L-Alanine	120	Niacinamide	1.0
L-Serine	150	Ca pantothenate	2.0
L-Cystine·HCl	75	Pyridoxal·HCl	1.0
L-Aspartic Acid	270	Thiamine·HCl	1.0
L-Glutamic Acid	315	i-inositol	1.0
L-Proline	115	Riboflavin	0.1
		B_{12}	0.002
Antibiotics, etc.:			
Methocel 15 cps	1000		
N.P.H. insulin	200 units/l		
Streptomycin	100 mg/l		
Penicillin	100,000 units/l		
Phenol red	10 mg/l		

II. MATERIALS AND METHODS

The experiments were conducted in 30-ml of medium contained in 100-ml serum bottles in a New Brunswick Gyrotory shaker at 35°C. HeLa, cat kidney, chick embryo, and L cells were obtained from stock cultures and grown in suspension using the LAH or the NEAA medium.*

The cultures were inoculated with virus by adding 10^8 mouse intracerebral LD_{50} ($MICLD_{50}$) per milliliter of an egg seed preparation of a virulent strain of VEE virus. A sample was obtained immediately after inoculation to determine the initial virus-inoculum concentration prior to the addition of the cells. The cells were then added to final concentrations of from 5×10^5 to 1.5×10^6 per ml and the virus cell mixture was incubated for 45 minutes on the shaker. The culture was then centrifuged, washed once, and resuspended in fresh medium. At this time a 0-hour sample for virus titration and a cell count was obtained. The cell concentrations were determined by counting cells unstained after treatment with 0.5 per cent trypan blue. The counting was performed at varying intervals, usually 24 hours apart.

Virus titers, determined by the Reed and Muench method, are expressed as a $MICLD_{50}$ obtained after the injection of 12- to 14-gram mice with 10-fold diluted samples.

III. OBSERVATIONS

A. VEE VIRUS

Figure 1 describes the replication of VEE virus in HeLa cultures grown in LAH, the growth of virus in the presence of 10 per cent calf serum, and the rate of virus inactivation in the presence of serum-free and serum-containing media.

The initial virus inoculum in the media was 10^8 $MICLD_{50}$ per ml. After an incubation of 45 minutes and one wash, virus concentration in the LAH, shown

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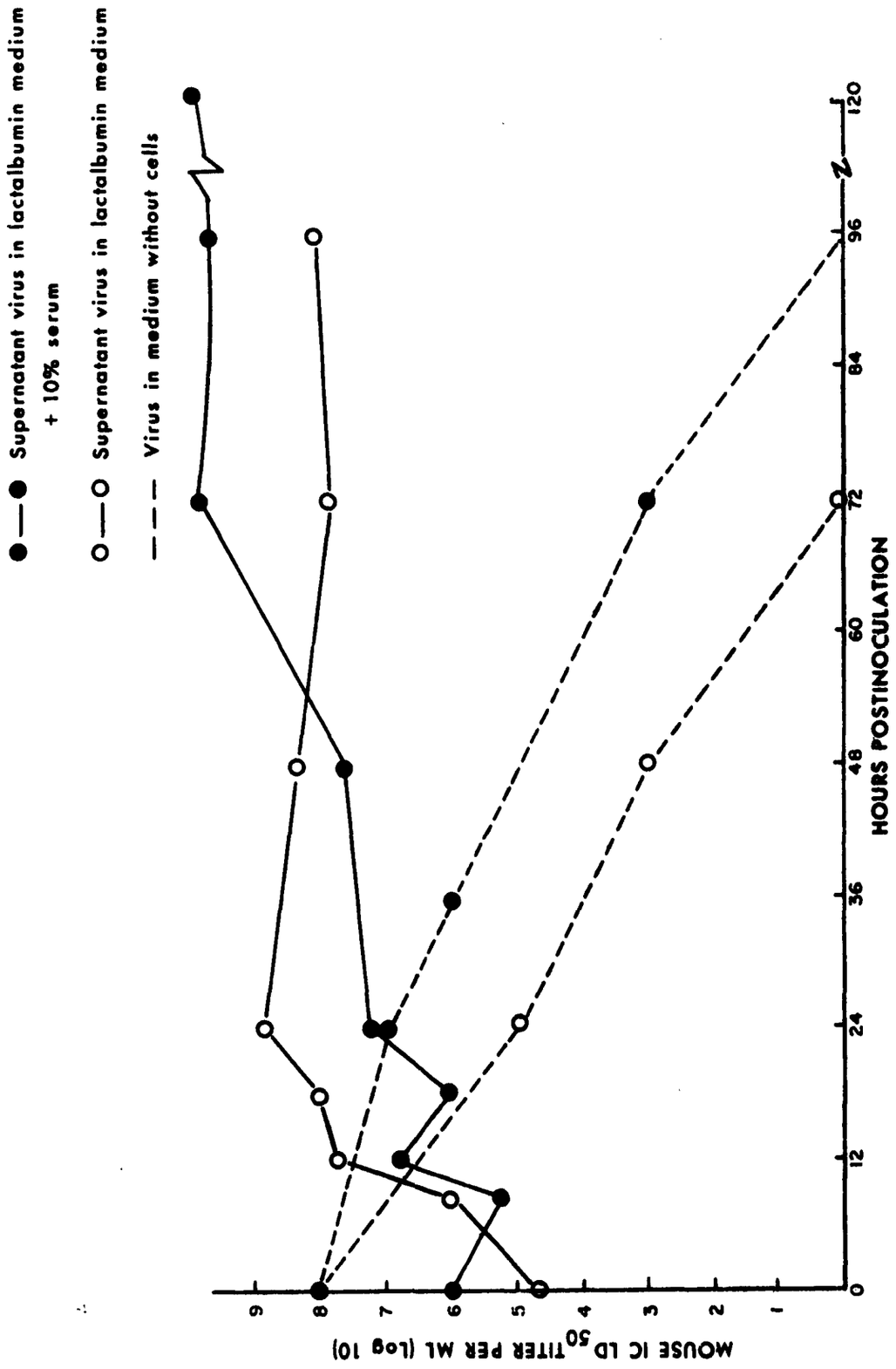

Figure 1. Growth of Venezuelan Equine Encephalomyelitis Virus in Serum-Free Suspension Cultures of HeLa Cells.

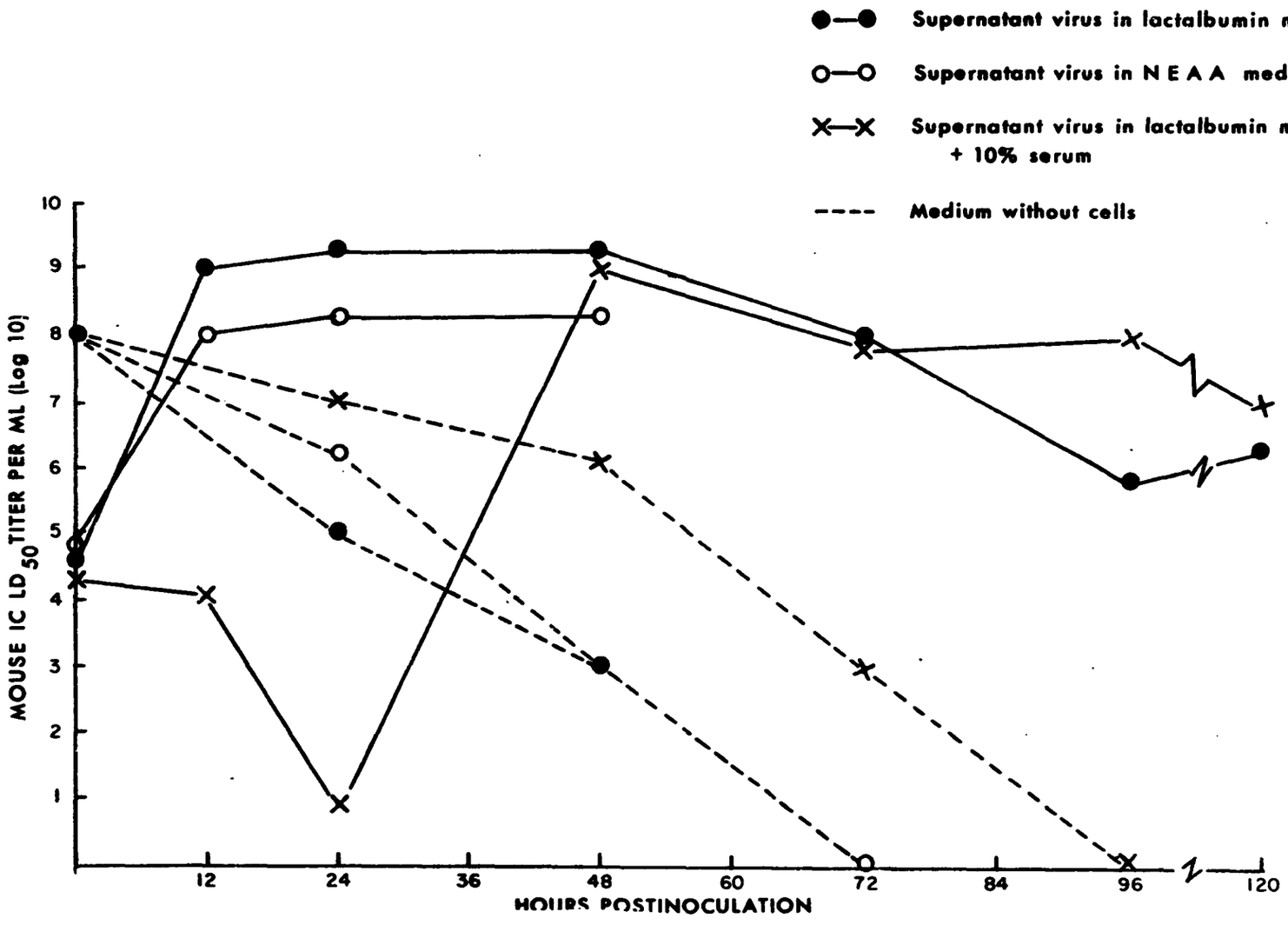

Figure 2. Growth of Venezuelan Equine Encephalomyelitis Virus in Serum-Free Cat Kidney Suspension Cultures.

as the open circles and solid line, beginning at 0 hour, was $10^{4.8}$ $MICLD_{50}$ per ml. A maximum titer of 10^{9} $MICLD_{50}$ occurred in about 24 hours. By the 96th hour, the titer had decreased about 1 log.

The closed circles and solid line represent virus titers in the supernatant of the serum-containing medium. Starting from 10^{6} $MICLD_{50}$ per ml at 0 hour, it did not reach the maximum titer of $10^{8.5}$ $MICLD_{50}$ per ml until 72 hours postinoculation. The virus remained at approximately the same level through 120 hours.

The broken lines represent the virus inactivation in cell-free media. The upper line represents the serum-containing medium, in which the virus titer did not disappear until the end of 96 hours. The lower broken line shows results in the serum-free lactalbumin medium, in which the virus titer completely disappeared by 72 hours.

In the first 48 hours the cells and supernatant fluids were titrated separately. After this time the cells and fluids were combined.

During the periods of observation, the cell-associated virus was found to be about 1/2 to 1 log lower than that in the supernatant fluid. At no time was it observed to be higher in the cells than in the fluid.

Both cultures initially contained a million cells per ml. Lysis of all but a small portion of the cell population occurred in the LAH by 72 hours. In contrast, the cell count in the serum-containing medium remained near preinoculation levels until 96 hours after which time approximately 60 per cent of the cells were found to have been destroyed. The delay in cell lysis, therefore, coincided with the delay in the production of maximal titers of virus in cultures to which serum was added. It might be added that this was a consistent finding among all cultures to which serum was introduced. All uninfected cultures continued to show cell multiplication during the duration of the experiment.

The growth of VEE virus in the cat kidney cell line is shown in Figure 2. This is a fibroblast-like cell, originally isolated in our laboratory. The initial viral inoculation was 10^{8} $MICLD_{50}$ per ml, and after the 45-minute incubation period and one wash, the 0-hour titer was 10^{4} to 10^{5} $MICLD_{50}$ per ml. The virus inactivation curve presented here shows that the viral stability in lactalbumin and defined medium are essentially the same, with the virus becoming undetectable in 72 hours.

Virus growth in NEAA, represented by open circles and a solid line, and the virus growth in LAH represented by closed circles and a solid line, resulted in maximal virus titers in 24 hours. Titers in the LAH medium were 10^{9} $MICLD_{50}$ per ml; those in the NEAA medium were 10^{8} $MICLD_{50}$ per ml.

The serum-containing medium did not show a maximum titer until 48 hours postinoculation, at which time it contained 10^9 $MICLD_{50}$ per ml. This culture continued to be infected for an extended period of time. Observations indicated that after the 33rd day the virus lost its ability to infect mice by the intraperitoneal route and, in fact, immunized the mice against challenge with fully virulent virus. This phenomenon will be described in more detail later in this paper.

The cell population responded to the virus in the expected manner, with complete lysis in 4 to 5 days except in the serum-containing medium, in which cell multiplication continued without abatement.

Figure 3 shows VEE virus growth in L cells in NEAA, LAH, and serum-containing media. The usual patterns of growth were obtained with the serum-free medium. The maximal levels of virus were 10^9 $MICLD_{50}$ per ml with LAH (closed circles) and 10^8 $MICLD_{50}$ per ml with the NEAA medium (triangles) in 24 hours. Virus growth in serum-containing medium was considerably delayed in this cell line and did not reach its maximum titer of 10^8 $MICLD_{50}$ per ml until 4 days postinoculation.

The previous studies suggested that virus would be produced for intervals considerably beyond the early postinoculation intervals. For this reason, these cultures were observed daily for 16 days, resulting in the disclosure of secondary peaks of viral growth at 11 to 12 days. Shortly after this time, the cultures in the LAH and the serum-containing media underwent complete cell lysis and were terminated. The culture in the NEAA medium, however, continued to demonstrate a high level of cell proliferation and a persistent production of virus for a total of 115 days. The virus that was obtained at the late intervals was found to vary considerably from that produced in the same culture at the early postinoculation intervals. For example, virus produced at the late interval was no longer lethal for mice by the intraperitoneal route, but it was highly immunogenic.

Additional experiments designed to elucidate factors associated with the chronic infection of these cultures were carried out. Space does not permit a detailed description of the results but they are summarized as follows: The interval of viral growth in the infected cultures was clearly divisible into two phases, an acute and a chronic, separated by a transitional phase. During the acute phase, which lasted from 1 to approximately 96 hours, large amounts of virulent virus were produced, generally in association with the destruction of the majority of the cells. This may then be succeeded by a relatively variable interval of transition that lasts from the fifth to the tenth day. The interval is characterized by a repopulation of the culture by the surviving cells, a decline in virus production, and a complete resistance of the cells to superinfection with virulent virus. The final or chronic phase of the infection usually begins after the eighth or ninth day and can persist for indefinite lengths of time unless the culture is terminated deliberately or becomes contaminated. It is characterized by the

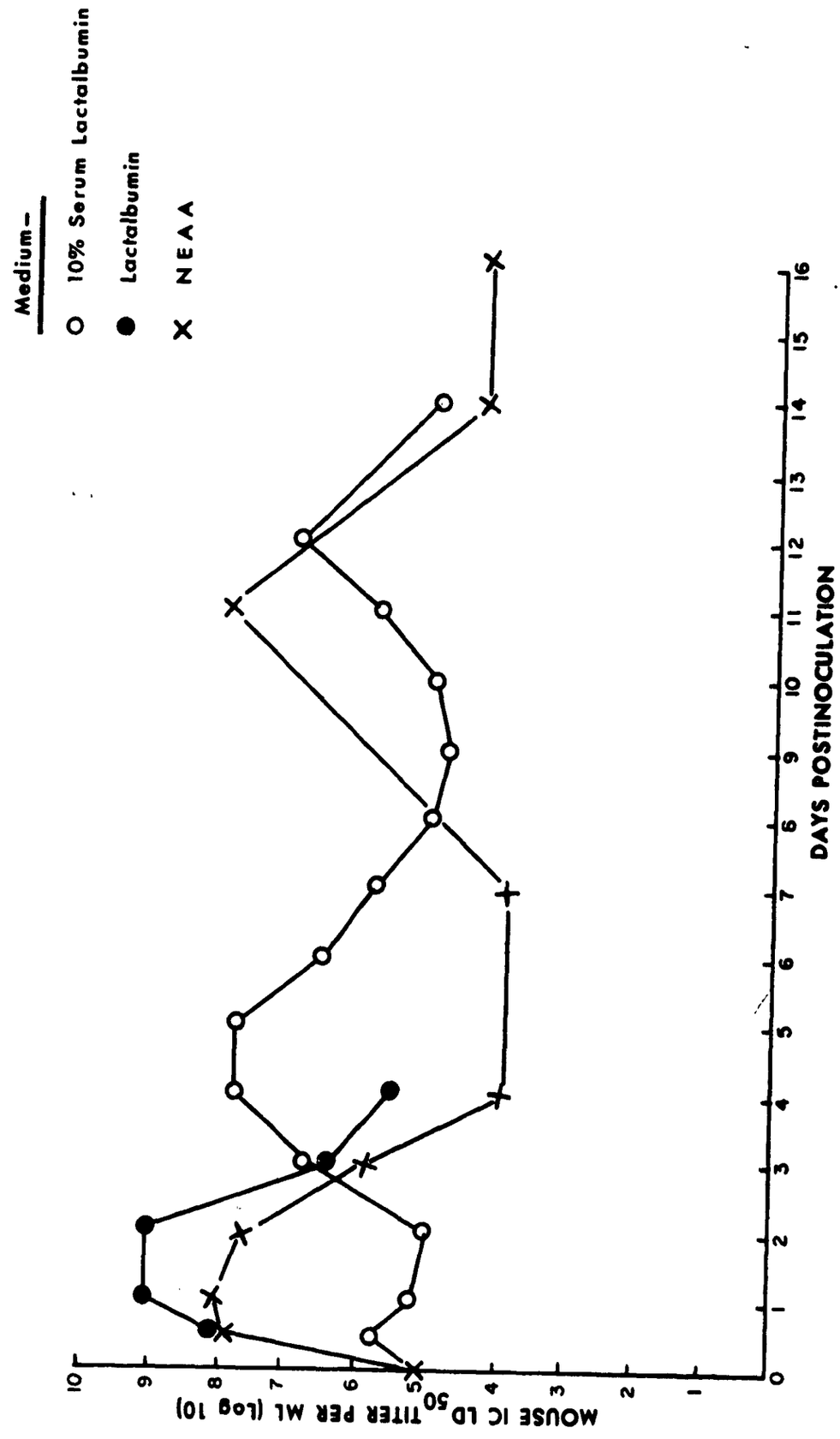

Figure 3. Growth of Venezuelan Equine Encephalomyelitis Virus in Serum-Free L Cell Suspension Cultures.

proliferation of cells that continue to be resistant to superinfection, the production of virus at levels between 10^2 and 10^4 $MICLD_{50}$, and a slow but observable selection of avirulent virus particles. As examples of this, at approximately eight days the virus became nonlethal for rabbits, nonlethal for mice between two to three weeks, nonlethal for guinea pigs at 72 days, and nonlethal for hamsters at 112 days postinoculation when tested by the intraperitoneal route. The newly selected avirulent population was genetically stable and highly immunogenic.

B. YELLOW FEVER VIRUS

The results of studies on the production of yellow fever virus in serum-free cultures is demonstrated in the final two figures. Figure 4 shows that the growth of the virus in the chick embryo cell line propagated in the LAH and LAH plus 10 per cent calf serum cells resulted in maximal titers of 10^7 and $10^{6.2}$ $MICLD_{50}$ respectively, six to eight days postinoculation. Some evidence of submaximal yields of virus appeared at the second day in both media. This was more readily apparent, however, in the culture containing LAH and calf serum. It is noteworthy that the maximal yellow fever virus yield was not delayed in medium with serum as it was in the case mentioned earlier during tests with VEE virus. Whether the serum was responsible for the temporary decline in virus production that was found three to five days postinoculation of the culture, however, is presently undetermined.

Figure 5 shows the results of propagating yellow fever virus in HeLa cultures grown in LAH and NEAA. The virus was produced to approximately the same maximal yields at three and four days of $10^{7.2}$ to $10^{7.4}$ $MICLD_{50}$ per ml. In contrast to the results of VEE virus production, cells that supported the growth of yellow fever virus failed to lyse or demonstrate any obvious signs of pathology.

An additional phenomenon of considerable interest was disclosed when the virulence of virus grown in chick embryo was compared with that of virus grown in HeLa cells. The fact that each product assayed to approximately the same titer in mice has already been shown. Tests with these products in monkeys, however, revealed a wide divergence of virulence. The chick embryo cell product after only one passage was nonlethal for monkeys when given in doses of 1000 $MICLD_{50}$ by the respiratory route. It was immunogenic, however, at doses of 10 $MICLD_{50}$ and above. In contrast, between 5 and 10 $MICLD_{50}$ of the HeLa cell product were sufficient to produce lethality by the respiratory route. Infection with this preparation was almost always associated with lethality.

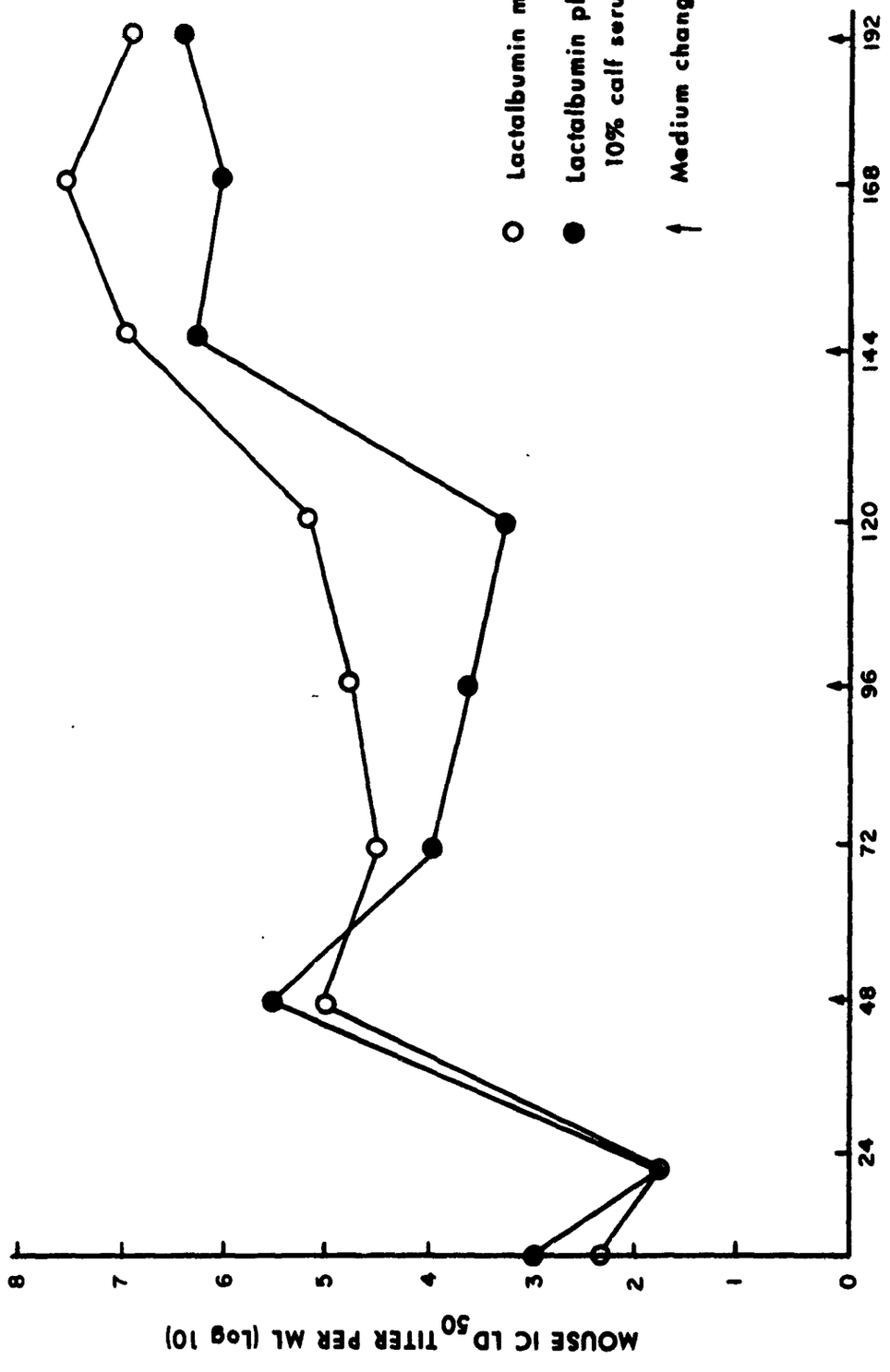

Figure 4. Growth of Yellow Fever Virus in Serum-Free Chick Embryo Suspension Cultures.

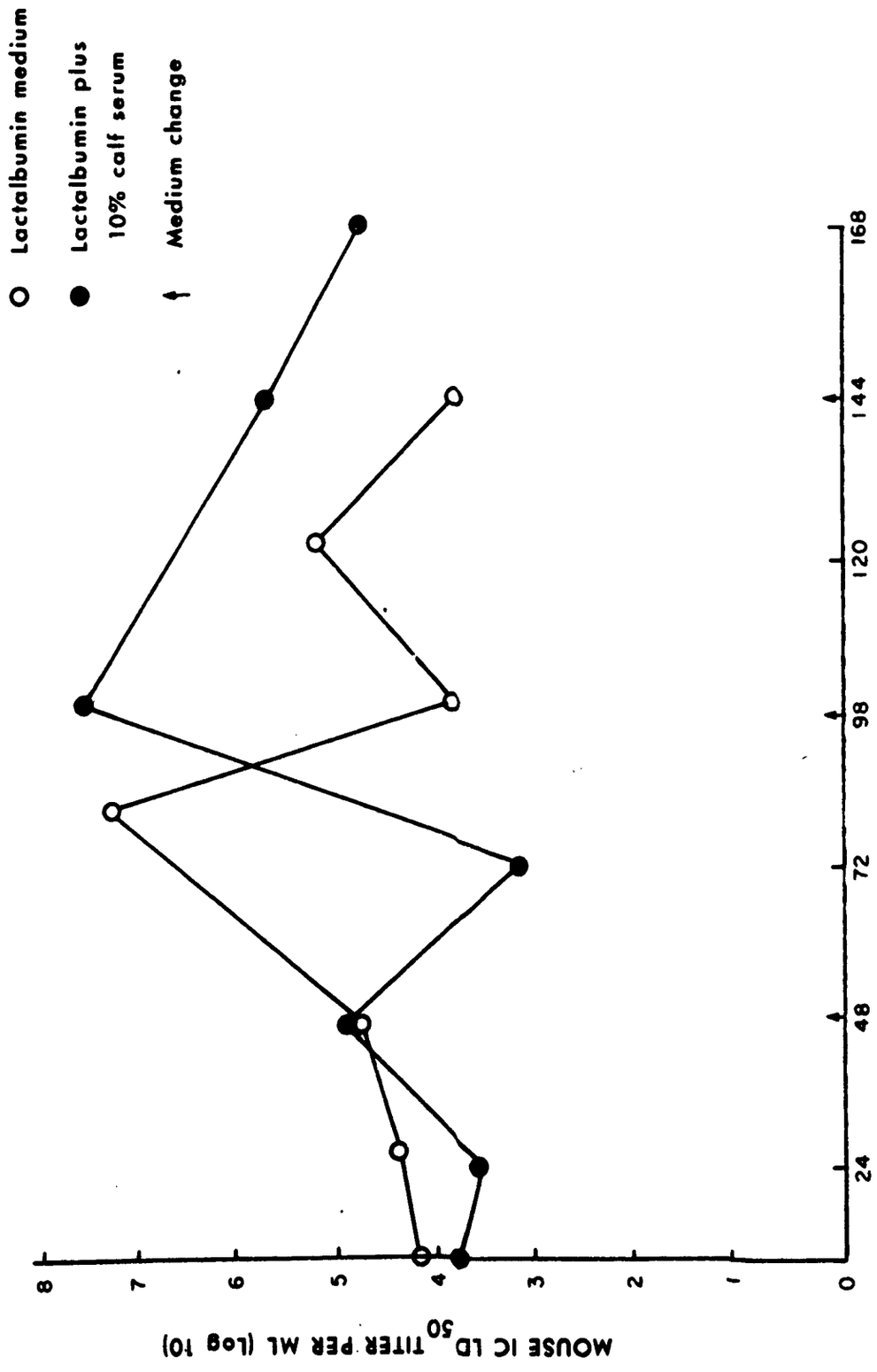

Figure 5. Growth of Yellow Fever Virus in Serum-Free HeLa Suspension Cultures.

IV. CONCLUSION

In summary, the ability of animal cell suspension cultures grown in two types of serum-free media to support the replication of VEE virus has been demonstrated. The interval of viral growth in the infected cultures was clearly divisible into 2 phases, acute and chronic, separated by a transitional phase. During the acute phase, large amounts of virulent virus were produced, generally in association with the destruction of the large majority of the cells. Titers of 10^8 and 10^9 $MICLD_{50}$ per ml within 24 hours postinoculation were obtained in HeLa, cat kidney, and L cells grown in either lactalbumin hydrolyzate medium or a defined medium containing essential and/or nonessential amino acids. Only slight individual differences in virus growth were observed among the different cell lines grown without serum. The addition of 10 per cent calf serum, however, altered the replication pattern of the virus. This was made manifest as a delay in the attainment of the maximum titer and in the appearance of cell lysis resulting from virus growth. The observed occurrences of a chronic state of infection in the cultures and the step wise decline in virulence of the chronically produced virus for various laboratory hosts was described. Yellow fever virus replicated in HeLa and chick embryo cell lines. Titers of approximately 10^7 $MICLD_{50}$ per ml were obtained in three to four days in both cultures. A widely differing qualitative response was detected, however, because of the rapid selection of an immunogenic population in the chick embryo cells that was nonlethal for monkeys. In contrast, HeLa cultures produced a highly virulent population that rarely failed to infect without causing lethality in monkeys.

These data indicate that serum-free suspension systems may provide a valuable means for growing viruses by researchers engaged in a wide variety of disciplines. The production of vaccines free of foreign antigenic protein, qualitative biochemical determinations of nutritional requirements, and inexpensive methods for producing viruses that are incapable of growth in conventional host systems are but a few of the areas in which serum-free suspension systems can play a highly important role.